Improvement and Assessment of Enzyme-Linked Immunosorbent Assay to Detect Low FK506 Concentrations in Plasma or Whole Blood within 6 Hours

Pierre E. Wallemacq, Ilham Firdaous, and Alexandre Hassoun

FK506, a promising new immunosuppressant, is currently under clinical investigation. Because dose-dependent toxicity is possible, blood concentrations of FK506 should be monitored. We improved the original ELISA of FK506 by shortening the incubation time. With some modification of materials, results are obtained within 6 h instead of 2 days, with similar or even better precision. Internal and external quality-control programs showed that our results correlated satisfactorily both with values determined by the original method and the theoretical expected values. Either plasma (detection limit 0.1 µg/L) or whole-blood (detection limit 1 µg/L) samples can be used. The sensitivity of the method makes it particularly useful for accurate pharmacokinetic studies or measurement of low blood concentrations. Twenty-four drugs and nine biological variables showed no significant interference on the assay. Study of the concentration- and temperature-dependent distribution of FK506 shows that the drug is largely bound to erythrocytes (ratio of blood to plasma concentrations is 10–40); as the erythrocytes become saturated, more of the drug is found in the plasma. Plasma concentrations may vary according to the blood temperature. We conclude that whole blood should be used for FK506 monitoring, as it is for monitoring cyclosporine.

Indexing Term: monitoring therapy

FK506 (tacrolimus) is a novel 23-membered macrolide lactone discovered in 1984 by workers from Fujisawa Pharmaceuticals in Japan. It is a neutral and hydrophobic compound with the molecular formula C_{44}H_{66}NO_{12}·H_{2}O (821 Da) (Figure 1). The action of this fungal immunosuppressant is similar to that of cyclosporine. The first experimental data on FK506 were published in 1987 (1, 2), and first clinical trials started 2 years later in Pittsburgh. Particularly encouraging results were obtained in pediatric recipients of liver allografts (3), suggesting a lower risk/benefit ratio than is found with other immunosuppressants. However, recent data show that, despite its recognized therapeutic properties, FK506 has some toxicity, which underscores the need for careful monitoring of blood concentrations. The whole-blood concentrations of FK506 that lead to toxic events are not clearly defined. The drug is currently under clinical investigation, and whole-blood or plasma concentrations are determined by a sensitive enzyme-linked immunosorbent assay (ELISA) developed by Tamura et al. (4). For this ELISA a mouse monoclonal antibody to FK506 is used. The original method can be briefly summarized as follows. Anti-mouse IgG is adsorbed onto a 96-well flat-bottomed plate and incubated overnight at 4 °C. A 100-µL sample of plasma is pretreated in acidic solution, and FK506 is extracted with a C_{18} Sep-Pak solid-phase column. Methanol is used to elute the drug, and dry residues are reconstituted with peroxidase-labeled FK506. Reconstituted sample and monoclonal antibody are placed in each precoated well, and competitive binding occurs overnight at 4 °C. After a washing step, the activity of the bound FK506-peroxidase conjugate is determined by measurement of the increase in absorbance at 492 nm after a short incubation with o-phenylenediamine substrate. The detection limit of this assay is 0.1 µg/L of plasma. This method is currently used in most centers, but is time-consuming (2 days), tedious, and relatively imprecise, with reported interassay CVs ranging from 15% to 27% (5) and from 13% to 23% (6).

The purpose of the present study was to improve the precision and accuracy of the assay and decrease the incubation time. By reducing the assay time, we could provide a clinically useful result on the same day that blood is drawn. We assessed the performance of the modified assay, using both internal quality controls and an external international quality-control program. We investigated the effect of blood temperature on the distribution of FK506. In addition, we determined how the duration and temperature of the incubation affects the performance of the assay. We tested numerous drugs and biological conditions such as hyperlipidemia and hyperproteinemia to determine which would interfere in the assay. Finally, plasma concentrations of FK506 were correlated with whole-blood concentrations, and

Fig. 1. Chemical structure of FK506

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Received September 1, 1992; accepted November 13, 1992.
the influence of hematocrit and of a saturation process
was studied.

**Materials and Methods**

Aliquots of FK506 in methanol (100 μg/L), peroxy-
dase-labeled FK506, and mouse monoclonal anti-FK506
antibody were kindly provided by Fujisawa Pharmaceu-
ticals Co. Ltd., Osaka, Japan, and distributed by BCO,
Center for Research, Breda, The Netherlands. Poly-
clonal anti-mouse IgG was from Atlantic Antibodies, Still-
water, MN. We used standard glass test tubes (60 × 12
mm) for all preparation and extraction steps. Phos-
phate-buffered saline (PBS) was from Gibco, Paisley,
Scotland; o-phenylenediamine and bovine serum albumin
(BSA) were from Sigma, St. Louis, MO; 96-well
flat-bottomed microtiter plates (MaxiSorp F8 Immuno
Module) were from Nunc, Roakilde, Denmark; C18
3-mL Bond-Elut Columns were from Analytichem, Harbor
City, CA; glacial acetic acid and hydrochloric acid for
analysis were from Panreac, Barcelona, Spain; and
HPLC methanol was from Labscan Ltd., Dublin, Ire-
land.2 We performed solid-phase extraction and evap-
oration with a Vac-Elut SPS 24 (Analytichem), a Ther-
molyne Dri Bath DB 28120-26 (for 60 samples)
maintained at 40 °C, and a system of needles connected
to a compressed air source. Plates were agitated on an
IKA Schatttler MTS 2. We measured absorbance with a
Vmax Microplate reader and evaluated the data ob-
tained with Softmax, version 2.01, both from Molecular
Devices, Menlo Park, CA.

**Interference Study**

Table 1 lists the 24 drugs and 9 endogenous com-
pounds that we tested for interference with the assay.
We tested plasma samples that contained the drugs (one
drug per sample) at supratherapeutic concentrations
(including drugs that might be coadministered with
FK506 structurally related agents), or one of the endog-
enuous compounds. Each interferent was tested twice, in
duplicate samples.

**Preparation of Quality-Control Samples**

Drug-free human plasma and whole blood were ob-
tained from the blood bank to prepare control samples
fortified with FK506 (0.5 and 1.5 μg/L in plasma; 10 and
30 μg/L in whole blood). Aliquots of these samples were
stored frozen until used to determine accuracy and
precision.

**Plate Preparation**

Freshly prepared solution of polyclonal anti-mouse
antibody (200 μL, 2.5 mg/L in PBS) is added to each
well. The plate is covered with Parafilm and agitated on
a plate rotator at 4 °C. The plates should be coated 24 h
before the assay is performed. However, we found
that coated and covered plates are stable up to 3 days at 4 °C.

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**Table 1. Drugs and Biological Analytes Tested to Assess Interference in the ELISA**

<table>
<thead>
<tr>
<th>Drug and analyte</th>
<th>Plasma concentration</th>
</tr>
</thead>
<tbody>
<tr>
<td>Erythromycin</td>
<td>2.5 and 5 mg/L</td>
</tr>
<tr>
<td>Amikacin</td>
<td>30 mg/L</td>
</tr>
<tr>
<td>Vancomycin</td>
<td>50 mg/L</td>
</tr>
<tr>
<td>Gentamicin</td>
<td>10 mg/L</td>
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<tr>
<td>Tobramycin</td>
<td>10 mg/L</td>
</tr>
<tr>
<td>Nefidimicin</td>
<td>10 mg/L</td>
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<tr>
<td>Theophylline</td>
<td>20 mg/L</td>
</tr>
<tr>
<td>Phenobarbital</td>
<td>40 mg/L</td>
</tr>
<tr>
<td>Valproic acid</td>
<td>75 mg/L</td>
</tr>
<tr>
<td>Carbamazepine</td>
<td>12 mg/L</td>
</tr>
<tr>
<td>Phenytoin</td>
<td>25 mg/L</td>
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<tr>
<td>Primidone</td>
<td>16 mg/L</td>
</tr>
<tr>
<td>Ethosuximide</td>
<td>100 mg/L</td>
</tr>
<tr>
<td>Acetaminophen</td>
<td>100 mg/L</td>
</tr>
<tr>
<td>Salicylate</td>
<td>500 mg/L</td>
</tr>
<tr>
<td>Digiotoxin</td>
<td>40 μg/L</td>
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<tr>
<td>Dibucin</td>
<td>3 μg/L</td>
</tr>
<tr>
<td>Diclofenamide</td>
<td>4 mg/L</td>
</tr>
<tr>
<td>Procolaine and N-acetylprocainamide</td>
<td>10 and 15 mg/L</td>
</tr>
<tr>
<td>Quinidine</td>
<td>4 mg/L</td>
</tr>
<tr>
<td>Flecainide</td>
<td>1 mg/L</td>
</tr>
<tr>
<td>Lidocaine</td>
<td>5 mg/L</td>
</tr>
<tr>
<td>Methyldprodinsalocine</td>
<td>5 mg/L</td>
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<tr>
<td>Cyclosporine</td>
<td>750 and 1500 μg/L</td>
</tr>
<tr>
<td>Bilirubin</td>
<td>300 mg/L</td>
</tr>
<tr>
<td>Protein</td>
<td>88 g/L</td>
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<tr>
<td>β-Lipoprotein</td>
<td>2550 mg/L</td>
</tr>
<tr>
<td>α1-Acid glycoprotein</td>
<td>1810 mg/L</td>
</tr>
<tr>
<td>Triglycerides</td>
<td>4220 and 8640 mg/L</td>
</tr>
<tr>
<td>Rheumatoid factor</td>
<td>1:512 (Waaler-Rose)</td>
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<tr>
<td>Gammopathies</td>
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<tr>
<td>IgA</td>
<td>2.3 g/L</td>
</tr>
<tr>
<td>IgM</td>
<td>0.2 g/L</td>
</tr>
<tr>
<td>IgG</td>
<td>2.6 g/L</td>
</tr>
</tbody>
</table>

All assays were performed twice in duplicate samples.

**Plasma Sample Preparation and Solid-Phase Extraction**

All standard, control, and patients’ samples are ana-
yzed in duplicate; 100 μL of plasma, either from the
blood bank (for calibration samples) or from patients, is
added to 1 mL of 0.1 mol/L HCl with 10 μL of standard
solution (FK506 in methanol) or methanol blank. Cal-
ibration samples have final FK506 concentrations of 10,
5, 2.5, 1.25, 0.625, 0.312, and 0.156 μg/L of plasma. We
prepared C18 columns by washing them first with 2 mL
of methanol and then with 2 mL of 0.67 mol/L aqueous
acetic acid under reduced pressure. Next, 1 mL of the
diluted and vortex-mixed plasma specimen is allowed to
pass through the cartridge, which is subsequently
washed with 2 mL of 0.67 mol/L acetic acid. FK506 is then eluted with 2 mL of methanol. The eluate is
collected and evaporated to dryness. Recycling the C18
Bond-Elut columns is possible after adequate washing
and preparation (3 mL of methanol and 2 mL of 0.67
mol/L acetic acid).

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2 Nonstandard abbreviations: PBS, phosphate-buffered saline; BSA, bovine serum albumin; BTPBS, PBS enriched with BSA and
Tween 20.

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Whole-Blood Sample Preparation and Solid-Phase Extraction

A similar procedure is followed for whole-blood determinations, but 25 μL of whole blood is added to 2 mL of 0.1 mol/L HCl with 25 μL of the standard solution or methanol blank. Calibration samples have final FK506 concentrations of 75, 50, 37.5, 25, 12.5, 6.25, and 3.125 μg/L of whole blood. Aliquots (1 mL) of the prepared samples are passed through the columns. All other steps are similar to the plasma preparation described above.

Assay Procedure

After incubation, on the day of analysis, the wells are washed with PBS and then saturated with 300 μL of BT-PBS (PBS enriched with 0.16 mmol/L BSA and 0.5 mL Tween 20) for 30 min at room temperature. Dry residues are reconstituted with 200 μL of a solution of peroxidase-labeled FK506 (diluted 1:300 in BT-PBS), and vortex-mixed three times. After aspiration of the residual BT-PBS, 180 μL of each reconstituted sample is added, one sample to each well. Next, 50 μL of the monoclonal anti-FK506 antibody (20 μg/L in BT-PBS) is added to each well. During the incubation period, plates are kept at room temperature and gently agitated while the competitive binding process is occurring. After an incubation of 2 h, the plate is washed first with PBS plus Tween 20 (0.5 mL/L) solution and then with PBS to remove unbound FK506. Immediately, 200 μL of the substrate solution (20 mg of o-phenylenediamine, 10 μL of 8.8 mol/L H₂O₂ in 10 mL of phosphate-citrate buffer, pH 5.4) is added to each well. The activity of the bound FK506-peroxidase conjugate is measured by the increase in absorbance at 492 nm after 15 min of incubation in the dark at room temperature, stopped by addition of 50 μL of 2 mol/L H₂SO₄. The standard curve is plotted by using a four-parameter logistic curve fit, and unknowns are calculated from the regression line.

Results

Recovery from the FK506 solid-phase extraction is around 85%. Cost-effective recycling of the columns yields progressively lower recovery (loss of 10–15%), which should be acceptable if new and recycled columns are not mixed. Correlation coefficients analyzed by the four-parameter curve-fitting method typically range from 0.995 to 1.000. Measurements of absorbance vary from day to day, with the zero and 10 μg/L standards in the range of 1.100 ± 0.020 (SD) and 0.990 ± 0.010 (SD) A, respectively. Calibration curves performed on the results of assays with different incubation conditions (overnight at 4 °C, 2 h at 20 °C, and 1 h at 37 °C) appear parallel but, with shorter incubation times, absorbance values are lower (around 8% and 20% lower absorbance values for the 2-h and 1-h incubation periods, respectively, compared with the overnight procedure). Standard deviations of the calibration curves are compared in Figure 2 for the three different incubation conditions. As displayed in Table 2, interassay CVs observed with plasma samples were 18% and 17% for expected FK506 concentrations of 1.5 and 0.5 μg/L, respectively. With whole-blood determinations, interassay CVs of 9% and 16% for 30 and 10 μg/L, respectively, were found. Intraassay CVs ranged from 6% to 9% for plasma and from 3% to 8% for whole-blood determinations. The modified method was also evaluated, with equally good results, by an external quality-control program organized by Fujisawa Pharmaceuticals. The assay requires 5–6 h to perform, and the detection limit is 0.1 μg/L in plasma and 1 μg/L in whole blood. We shortened the incubation phase to 2 h at room temperature (around 20 °C, maintained by air conditioning) and compared the results obtained with those obtained with the original incubation period (overnight at 4 °C). We found no statistical difference for patients' samples (Student's t-test, not significant; n = 24, slope 1.05, and r = 0.976), provided both incubating plates were agitated (Figure 3). If samples are incubated for 2 h without agitation, a statistically significant decrease (± 10%) in the measured concentrations (P = 0.001, n = 63) is found. A 1-h incubation at 37 °C leads to similar results, acceptable
Fig. 3. Comparison of the FK506 plasma concentrations obtained with the shortened incubation period (2 h at 20 °C) and with incubation overnight at 4 °C (n = 24)

in case of emergency. However, the observed decrease in absorbance (zero sample: around 0.850 A) may result in a slight loss of precision.

The temperature of the blood specimen is an important factor, which must be controlled. Maintained at 37 °C before centrifugation, plasma specimens from transplant patients display FK506 concentrations two to threefold higher than those of samples kept at 20 °C until centrifugation (n = 48, \( \overline{x} = 2.63 \pm 1 \)). Figure 4 shows (after 1 h of incubation of the blood sample at 37 °C) the FK506 residual fraction in plasma as a function of the time elapsed at room temperature before centrifugation. An equilibrium seems to be reached after 30 min.

None of the drugs and clinical biochemistry variables tested (Table 1) displayed significant interference in the assay at their respective ranges of concentration (results \( \leq 0.1 \mu g/L \) plasma).

We compared 105 plasma (y) and whole-blood (x) concentrations obtained from a pediatric liver transplant population. The ratio y/x varies considerably, from 10 to 40 (with extremes 4–90). No clear correlation could be found between such values (r = 0.417). Hematocrit (ranging from 0.231 to 0.44) was recorded for each blood specimen and correlated with the corresponding ratio y/x without success (n = 105, \( r = 0.171 \)) (Figure 5). The main factor influencing this ratio seems to be the FK506 concentration itself; low plasma concentrations correspond generally to a higher y/x ratio and, conversely, lower ratios are seen with high plasma concentrations. We believe this is the result of the saturation of red blood cells with FK506 as the concentration of FK506 increases.

Stability of the FK506 concentrations in plasma or whole blood (n = 10) maintained at room temperature was evaluated by ELISA during five consecutive days. A slight and progressive decrease (10–30%) was observed at the end of this period, both in plasma and whole blood.

**Discussion**

The aim of this work was to modify the original assay for FK506 so that results would be available on the day
of analysis and to improve, if possible, the accuracy and precision of the assay. The results described above indicate that the performance of the assay was improved (assay duration <6 h, absorbance SDs <0.03, and CVs <20%) by these minor modifications: an incubation time of 2 h at 20 °C instead of an overnight incubation at 4 °C, use of smaller volumes of methanol and acetic acid in the extraction steps, and the use of polyclonal antibody. We used Bond-Elut columns and Nunc-ImmuMonModule MaxiSorp plates. This assay allowed us to monitor our patients 7 days a week. Warty et al. (7) also shortened the incubation phase with success, but described a poorer correlation (r = 0.89) with the original procedure. During the first 6 months of 1992, our modified method was used for >1000 FK506 analyses in plasma and whole blood. Most of the plasma concentrations ranged from 0.2 to 1.2 μg/L, corresponding to whole-blood values of 4–25 μg/L. Correlations of data with clinical events (such as toxicity or rejection episodes) are currently being analyzed retrospectively. The monoclonal antibody used in this immunoassay seems to give results that correlate well with the suppression of the mixed lymphocyte reaction (8), and therefore it appears to be satisfactory for use in clinical monitoring. However, this antibody does have some cross-reactivity with metabolites of FK506. Friob et al. (9) described in transplant patients with hepatic dysfunction high concentrations (2–15 μg/L) of FK506 in plasma measured by ELISA, caused primarily by an active cross-reacting compound distinct from FK506, most probably a metabolite. The role played by these cross-reacting metabolites in immunosuppression or toxicity and the distribution of these metabolites in the blood require further elucidation. We describe similar FK506 distribution ratios (whole blood/plasma) and similar findings about the influence of temperature on FK506 plasma concentration measurements as were noted by other centers (6, 7, 10, 11). However, we did not observe any significant influence of the hematocrit on the FK506 distribution ratio as was reported elsewhere (11, 12). This contradiction is perhaps explained by the fact that the two groups cited above performed in vitro studies, using blood specimens fortified with FK506 that sometimes had hematocrit ranges as large as 0.10–0.70, whereas our purpose was to investigate the influence of the hematocrit in clinical situations such as in the actual range of hematocrit found in liver transplant patients. We believe that whole-blood measurements will replace plasma determinations for FK506, just as they did for cyclosporine.

In the near future, Abbott Laboratories will introduce an automated whole-blood MEIA (microparticle enzyme immunoassay) to measure FK506 on the IMx analyzer (13). This attractive method should be able to provide results within 45 min with a significant improvement in interassay and interlaboratory reproducibility. However, it has not yet been validated in clinical situations, and its sensitivity limit of 5 μg/L of whole blood might be inadequate for low-dose therapy or for accurate pharmacokinetics. Therefore, in the meantime, the modified ELISA remains a method of choice for these applications.

We thank Fujisawa Pharmaceuticals for their financial support and Joëlle Laporte and Marie-France Warmoes for excellent technical assistance.

References